Optimization of B-Glucan Production from Lower Fungi using Central Composite Design and its Biological Application

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ABSTRACT

Production of β-glucan is mainly used for cancer therapy. The most well-known are those occur in fungal grains such as cereal and other micro-organisms. The most important beta glucan from a nutritional and ingredient standpoint is that derived from fungal grain. Optimization of medium ingredients for the production of mycelial β-glucan from lower fungi in submerged fermentation and antioxidant activity of β-glucan was carried out.

Keywords
β-glucan, CCD, RSM, FTIR, Antioxidant activity

1. INTRODUCTION

The worldwide food industry uses 70,000 tonnes of polysaccharides per year as thickening agents, stabilizers and texturisers. As the emerging food products become more complex and diverse, the requirement for new and versatile additives is stronger. β-Glucans are a group of glucose polymers that form a fibrous structural extracellular matrix with in the cell walls of yeast [1] plants [2,3] and some micro organisms [4]. Fungi are currently of interest because they are a biologically rich source of various active substances. Polysaccharides have emerged as an important class of bioactive substances. Most of the polysaccharides mediating biological activities from mycelia were endopolysaccharides or exopolysaccharides [5]. Over the past 20 years there has been increasing interest in the large-scale production of fungal polysaccharides by submerged fermentation, notably in view of their diverse pharmacological activities [6,7]. Various β-glucans have been studied, and many exhibit bioactivities and confer health benefits [8,9]. With a view to develop β-glucan-containing value-added products, exploration of new sources of β-glucans and characterization of their origin-dependent bioactivities are of importance. Among the β-glucans of fungal polysaccharides, comprising intracellular-, structural-, and exocellular-polysaccharide, fungal exopolysaccharide are most favored by manufacturers, mainly due to its ease of recovery. However, up to now, no detailed investigations have been conducted on composition characterization and anti oxidative capacities of different β-glucan isolated from *Aspergillus* species. An overview of the literature on the production, characterization and medicinal properties of the fungal polysaccharides indicates that there are gaps in knowledge which remain to be explored and there is a lot of scope for isolating new polysaccharides from lower fungi with significant bioactivity. Keeping this perspective, the present investigation was designed to survey and screen media for β-glucan production and their biological activity.

2. MATERIALS AND METHODS
2.1 Preparation of β-glucan
The crude Beta-glucan was prepared according to the reported method with some modifications [10]. Briefly, the mycelia of lower fungi were harvested from the liquid medium by filtering through a filter paper, washed three times with distilled water, and dried at 60°C to a constant weight. The dry weight (DW) of the mycelia was determined in terms of g/100mL. The dry mycelia were ground using a domestic blender, extracted 3 times with distilled water at 90° C for 2 h each, and then centrifuged at 5000 rpm for 20 min. The supernatants were combined and reduced pressure. The resulting concentrate was mixed with three times volume of absolute methanol, stirred vigorously and kept overnight at 4°C. The precipitate was collected by centrifugation at 5000 rpm for 20 min, washed twice with acetone and ether respectively, and then dried. The resulting crude β- glucan was also estimated in terms of g/mL (DW).

2.2 Central Composite Design and Response Surface Methodology
The levels of the significant parameters and interaction effects between Medium-3 and the fungus *Aspergillus niger* was used for the production of β-glucan components which influence significantly the β-lucan production were analyzed and optimized by using a central composite design in response surface methodology. The experimental design was carried out by using "Stat-Ease Design-Expert" software (version 8.1, Stat-Ease Corporation, USA). The four independent factors were investigated at five different levels (-2, -1, 0, $+1$, $+2$). The response Y (yield of β-glucan) was analyzed by using a second order polynomial equation in four independent variables and the data were fitted into the equation by multiple regression procedure.
The model equation for analysis is given below Eq.

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X^2_i + \Sigma \beta_{ij} X_i X_j$$

where Y is the predicted response, X_i, X_j represent the independent variables which influence the response variable Y, and β_0, β_i, β_{ii}, and β_{ij} represent the offset term, the ith linear coefficient, the ith quadratic coefficient and the ijth interaction coefficient, respectively. "Design-Expert"8.1 was used for regression and graphical analyses of the data obtained. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The student's t-test permitted the checking of the statistical significance of the regression coefficient, and the Fischer's test determined the second-order model equation. The quality of the fit of the polynomial model equation was given by the coefficient of determination (R2). The optimum concentration of the variables were calculated from the data obtained by using the

response surface regression procedure of the SAS statistical

2.3 FTIR analysis of β-glucan

β-Glucan were analyzed by FTIR-JASCO 4100 Spectrophotometer. IR spectra of the compounds were recorded for 400-4000 cm-1 in KBr pellet using a FT-IR spectrophotometer.

2.4 Antioxidant activity of polysaccharides on DPPH radical scavenging capacity

The antioxidant activity of β-glucan were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH method [11] as modified [12]. A methanolic solution (0.1ml) of sample extract at various concentrations was added to 3.9 ml (0.025gL^{-1}) of DPPH solution. The decrease in absorbance at 515nm was determined continuously recorded in a spectrophotometer for 16 minutes. The decrease in the absorbance depends on the concentration of the antioxidant and the radical, the molecular structure of the antioxidant, and its kinetic behavior. The scavenging effect (decrease of absorbance at 515nm) was plotted against the time and percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 16 min duration as follows.

$$\text{Percentage of DPPH activity} = \frac{\text{control OD- sample OD}}{\text{control OD}} \times 100$$

3. RESULTS

3.1. Isolation and identification of fungal strain

The strains were isolated from Rhizoshere soil sample collected from the agriculture field of Palacode, Dharmapuri (Dt), Tamilnadu. Soil samples were serially diluted and were plated on Potato dextrose agar medium. The plates were incubated 72 hours at room temperature and the growth of fungal colonies was observed. The fungal colonies grown on these plates were individually sub cultured and the pure cultures of spore forming units were identified based on the colony morphology and with spore staining techniques. The individual spore forming colonies in Potato dextrose agar plates were predominantly observed to be *Aspergillus* sp. Among many isolates of genus *Aspergillus* four different colonies with different morphological characters were chosen for the studies. The organisms were identified by following standard protocols reveals that the four isolates belonging to the genus *Aspergillus* lies to *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Aspergillus terreus.* These organisms were further used in this study for the production of β-glucan.

3.2. Media optimization for synthesis of β-glucan

The yield parameters of β-glucan the *Aspergillus* strains were shown in Table 1. Among the four strains tested, *Aspergillus niger* produced maximum of 0.5 gms β-glucans in basal medium-3 from 0.78 gms of mycelial dry weight in contrast basal medium-4 yielded only 0.38gms of β-glucan from 1.11gms mycelia dry weight. Maximum β-glucan production was observed in basal medium-3 followed by basal medium-2, 4.

3.3. Central Composite Design and Response Surface Methodology

package (Version 8.1, SAS institute inc. NC. USA) Central Composite Design is powerful method for screening significant factor in the presence study, 14 runs were carried out to investigate the production optimization of mycelial dry weight and β-glucan using two different factors including a Carbon source (corn starch) and Nitrogen source (yeast extract). The mycelial dry weight varied greatly from 0.56gms to 1.28gms where as in β-glucan it was 0.09 gms to 0.51 gms under different combination of media components. Response surface methodology help in evaluation of relationship between the dependent (β-glucan yielded) variable and independent variables and predicted values of the biomass yield are shown. The accuracy of the model can be seen by the different between observed and predicted value. The co-efficient and the analysis of variance are presented in table. Fitness of the model was expected by the value of the determination co-efficient in the present β-glucan comes out to be 0.51 and 0.50 for *Aspergillus niger* high value of adjust co-efficient determination this adjusted 0.70 and 0.90 respectively indicate high significance of model (Fig. 2 and Fig. 3).

3.4. Antioxidant activity by DPPH free radical scavenging assay

The scavenging activity of β-glucan in various concentration (10-100μg/ml) was determined by DPPH assay and the results are shown in Table 2. Increase in β-glucan concentration increases the antioxidant activity at a maximum of 78.31 percentage at 100 μg/ml of concentration and minimum of 1.20% at 10 μg/ml concentration. Similar experiments was carried out with ascorbic acid which resulted in a maximum of 51.80% radical scavenging capacity at 100 μg / ml concentration and a minimum of 25.30% scavenging activity at 10 μg / ml concentration.

3.5. FTIR spectrum

Figure 1 shows the FTIR spectrum of β-glucan of the *Aspergillus niger* isolated from basal medium – 3 by Response surface methodology. The large absorption peak at 3852.5 – 3903.6 cm^{-1} was C-H stretching vibration of alkenes and asymmetrical O-H stretching absorption band at range of 3750 cm^{-1} was found to be OH stretching. Absorption peaks at 3396 and 3397 was N-H stretching of hetero aromatic compounds. C-H stretching peaks of methyl a group (Alkanes) was between 2852 – 2928 cm^{-1}. The absorption band between 2345.1– 2363.9 cm^{-1} was attributed to the carbonyl absorption bands in addition to an ammonium band. Absorption peak at1733.8cm^{-1} was C=O stretching vibrations of the aldehyde group and 1637.0 – 1653.4 cm^{-1} was NH bending vibration to the amide group. The band at 1559.4 cm^{-1} was N=O absorption bands of aliphatic nitroso compounds. The band at 1419.1 cm^{-1} was attributed to the CH bending vibrations to the alkenes group. Absorption peak of cyclohexane was found nearby 1457.5 cm^{-1} wavelength followed by symmetrical C-H bending vibration to the alkynes at the peak 1375.5 cm^{-1}. Absorption peak for 1-phenyl ethanol belonging to the secondary alcoholic group was observed at 1075.7 – 1078.9 cm^{-1} and peaks for phosphorus organic compounds were observed at 1164.7 cm^{-1}. The band at 668.6 – 669.3 cm^{-1} was C-H bending vibration to the alkynes group.

Table 1. Yield parameter of dry weight and β-glucan by lower fungi

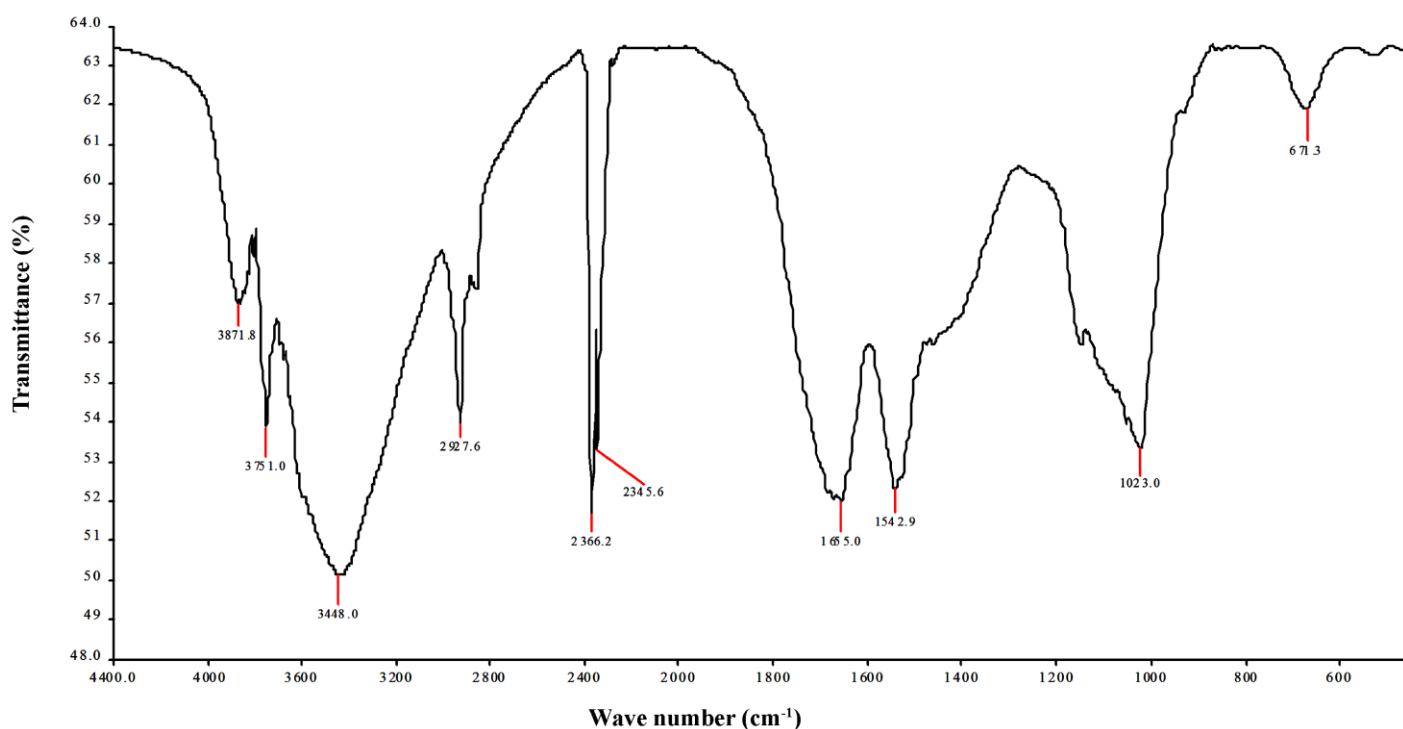| Medium	*Aspergillus niger*		*Aspergillus flavus*		*Aspergillus fumigatus*		*Aspergillus terreus*	
	Dry weight in grams	β-glucan in grams	Dry weight in grams	β-glucan in grams	Dry weight in grams	β-glucan in grams	Dry weight in grams	β-glucan in grams
Seed medium	0.73	0.10	0.60	0.21	0.38	0.21	0.70	0.21
Basal medium-1	0.92	0.09	0.72	0.09	0.28	0.17	0.52	0.24
Basal medium-2	1.6	0.45	1.5	0.3	0.43	0.24	0.66	0.21
Basal medium-3	0.78	0.5	0.89	0.27	0.40	0.15	0.49	0.32
Basal medium-4	1.71	0.38	0.87	0.31	0.31	0.03	0.81	0.12
Czapek Dox broth	0.57	0.09	0.57	0.10	0.15	0.07	0.40	0.09
MOPT broth	0.53	0.12	1.27	0.11	0.35	0.09	0.30	0.08
Fungal complete medium	0.23	0.04	0.22	0.08	0.07	0.04	0.31	0.06

Fig. 1. Infra Red Spectrum of β-Glucan isolated from *Aspergillus niger*

Table 2. 1,1-Diphenyl-2-picryl hydrazyl radical scavenging capacity of β-glucan of *Aspergillus niger* at different concentrations

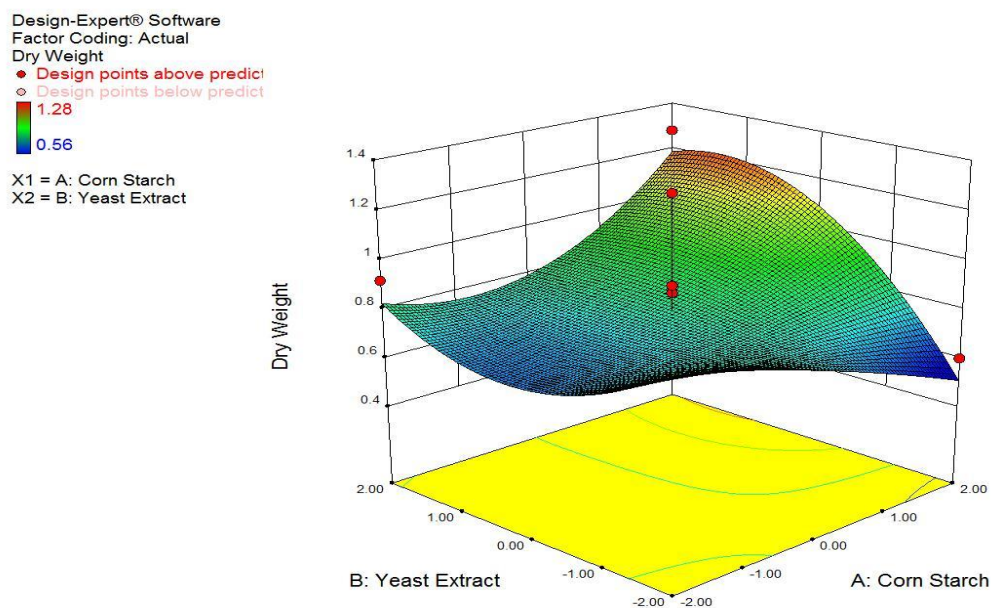| Concentration (μg/ml)	DPPH Radical Scavenging Activity of β-glucan (%)	DPPH Radical Scavenging Activity of Ascorbic acid (%)
10	1.20	25.30
20	3.61	27.71
30	8.43	28.91
40	15.66	31.32
50	22.89	32.53
60	27.71	37.34
70	37.34	40.96
80	39.63	43.37
90	50.60	49.39
100	78.31	51.80

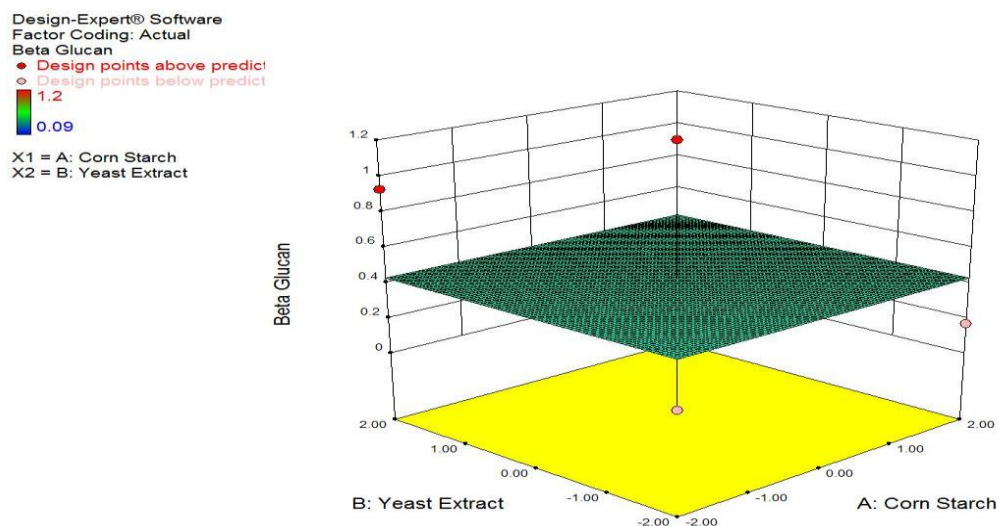**Fig. 2. Dry Weight Model Graph of *Aspergillus niger* in Basal M**

Fig.3. β-Glucan Model Graph of *Aspergillus niger* in Basal Media 3

Table 3. Response surface methodology *yield of β-glucan by* Aspergillus niger

RUN	FACTOR-1 (CORN STARCH)	FACTOR-2 (YEAST EXTRACT)	DRY WEIGHT IN GRAMS	β-GLUCAN WEIGHT IN GRAMS	pH LEVEL BEFORE INCUBATION	pH LEVEL AFTER INCUBATION
1	0	-2.83	0.60	0. 40	6.5	6.5
2	0.00	0.00	1.27	0.17	6.5	6.5
3	2.83	0.00	1.2	0.50	6.5	6.5
4	-2.00	-2.00	1.0	0.17	6.5	6.5
5	0.00	0.00	0.59	0.09	6.5	6.5
6	0.00	0.00	0.60	0.09	6.5	6.5
7	0.00	0.00	0.87	0.32	6.5	6.5
8	2.00	-2.00	0.60	0.17	6.5	6.5
9	0.00	0.00	0.56	0.19	6.5	6.5
10	-2.00	2.00	0.92	0.50	6.5	6.6
11	0.00	0.00	0.90	0.48	6.5	6.5
12	2.00	2.00	1.28	0.27	6.5	6.5
13	-2.83	0.00	0.57	0.15	6.5	6.5
14	0.00	2.83	0.70	0.51	6.5	6.5

4. CONCLUSION

RSM was used to estimate and optimize the polysaccharides production. All the independent variables, quadratic of all the independent variables had highly significant effects on the response values ($p < 0.03$). The optimal medium for β-glucan production was obtained through a central composite design in response surface methodology as follows Carbon source (corn starch 4.5 gm/l) and nitrogen source (yeast extract 0.4 gm/l). Under these conditions, the experimental yield of polysaccharides was 0.51 gms in the factor with preliminary media optimization experiments in basal medium-3 which was close with the predicted yield value (Table 3). It was also demonstrated that the administration of β-glucan could increase the antioxidant activity.

5. REFERENCES

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